

Glycosidized Low-Density Lipoprotein Downregulates Endothelial Nitric Oxide Synthase in Human Coronary Cells

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OBJECTIVES	We examined the hypothesis that low-density lipoprotein (LDL) that is both oxidized and glycosylated potentially downregulates the expression of endothelial nitric oxide synthase III (NOSIII) in human coronary endothelial cells.
BACKGROUND	Diabetes mellitus is accompanied by both oxidation and glycosylation of LDL, but the potential interaction of these processes or the pathophysiologic effects of these modified lipoproteins on arteries are poorly understood.
METHODS	Low-density lipoprotein was glycosidized in vitro, and Western and Northern blot analyses were used to investigate NOSIII expression in human coronary endothelial cells. Nitric oxide (NO) bioactivity was represented by both basal and bradykinin-stimulated cellular cyclic guanosine monophosphate accumulation and L-citrulline conversion from L-arginine. Nuclear run-on experiments were performed to study the transcription rate of nascent NOSIII messenger ribonucleic acid (mRNA).
RESULTS	Data showed a significant decrease in NOSIII expression after 24-h treatment with glycosylated low-density lipoprotein (glycLDL) and oxidized low-density lipoprotein (oxLDL). Accordingly, we observed a significant dose-dependent reduction in NO bioactivity ($p < 0.05$ to $p < 0.001$ vs. untreated cells, native low density lipoprotein [nLDL], glycLDL, and oxLDL). Glyc-oxLDL did not reduce the half-life of NOSIII mRNA or significantly enhance L-citrulline conversion. Nuclear run-on experiments showed that high doses of glyc-oxLDL can reduce the transcription rate of nascent NOSIII mRNA (densitometric analysis revealed a reduction of 25% [$p < 0.05$ vs. untreated cells, nLDL, and glycLDL] after treatment of cells with 300 $\mu\text{g/ml}$ glyc-oxLDL). The effects of glyc-oxLDL are not related to the higher levels of oxidative compounds in comparison to those of oxLDL.
CONCLUSIONS	These results indicate that glyc-oxLDL, per se, may influence signal transduction pathways involving NO-mediated regulatory signals and NOSIII activity in human endothelial cells. This phenomenon can adversely influence the evolution of clinical vascular complications, coronary heart disease, and atherogenesis in diabetic patients. (J Am Coll Cardiol 2002;40:1515-22) © 2002 by the American College of Cardiology Foundation

Biochemical modifications of low-density lipoprotein (LDL) that affect its composition can greatly enhance the injurious potential of LDL and its involvement in the pathogenesis of various disease states. Indeed, oxidized low-density lipoprotein (oxLDL) is prevalent in early and advanced human atherosclerotic lesions (1-3) and has been implicated in the cascade of events responsible for lesion formation and plaque rupture (1-5). Cell-culture experiments demonstrated that the deleterious effects of oxLDL include activation of several transcription factors in arterial cells (6-11) and altered bioactivity of endothelial nitric oxide synthase III (NOSIII) (12,13). Nitric oxide (NO),

which is derived from the guanidine nitrogen atoms of L-arginine through a reaction catalyzed by NOSIII (12), is a major contributor to the regulation of vascular tone, and it affects several pathways involved in atherogenesis and vascular dysfunction (13). An oxLDL-induced decrease in the expression of NOSIII might, therefore, lead to a decline in NO bioavailability and in the vascular protective and anti-atherogenic effects of NOSIII (13).

It is well established that proteins exposed to elevated glucose concentration, for example, in diabetic patients, may undergo glycosylation (14). Among these proteins, nonenzymatic glycosylation of LDL to form glycosylated low-density lipoprotein (glycLDL) involves the reaction of glucose with reactive amino groups and N-terminal amino acid residues to form a keto-amine linkage (Nomenclature Committee of International Union of Biochemistry 1984). Glucose can also react nonenzymatically with the amino groups of proteins to form advanced glycosylation end products (AGEPs) (15). Furthermore, the Maillard reaction (glycoxidation) involves a combination of both glycosylation and oxidation of proteins (16). The addition of glucose to

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Abbreviations and Acronyms

AGEP	= advanced glycosylation end products
cDNA	= complementary deoxyribonucleic acid
cGMP	= cyclic guanosine monophosphate
EDTA	= ethylenediamine-tetraacetic acid
EMSA	= electrophoretic mobility shift assay
glycLDL	= glycosylated low-density lipoprotein
LDL	= low-density lipoprotein
mRNA	= messenger ribonucleic acid
nLDL	= native low-density lipoprotein
NO	= nitric oxide
NOSIII	= endothelial nitric oxide synthase III
oxLDL	= oxidized low-density lipoprotein
RNA	= ribonucleic acid
SRE	= sterol-responsive element
TBARS	= thiobarbituric acid-reactive substances
TNBSA	= trinitrobenzenesulfonic acid

LDL incubated with metal ions (16–18) or oxygen radicals (18) *in vitro* yields a higher oxidation than incubation of LDL with oxidants alone. The pathophysiologic significance of this process is underscored by *in vivo* and *in vitro* evidence for glycooxidation of LDL in human atherosclerotic plaques (16,19). Although AGEPs may have been previously considered to be restricted to diabetes mellitus and aging (20), oxidation-related processes, *per se*, can lead to their formation, and AGEPs have been identified in atherosclerotic lesions without coexisting diabetes (21).

Molecular events promoted by oxLDL could be amplified by coexisting glycooxidation. Indeed, in addition to facilitating atherogenesis, the glycosylation process may also affect vascular function. Activation of the receptor for AGEPs (21) and quenching of NO linked to defective endothelium-dependent vasodilation in experimental diabetes (22) may constitute a mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. These events, together with glycosylation and oxidation of LDL, might induce sustained vascular dysfunction and coronary heart disease through alterations of NOSIII expression. Accordingly, the goal of the present study was to explore the mechanisms by which glycLDL and glyc-oxLDL modulate NOSIII in human coronary endothelial cells.

METHODS

Preparation of human glycLDL and oxidation. Low-density lipoprotein was isolated from the plasma of healthy volunteers (2 mmol/l ethylenediamine-tetraacetic acid [EDTA] and 1,000 UI/100 ml aprotinin) by ultracentrifugation in a KBr gradient, as previously described (23). Proteins were assayed by Lowry et al. (24). The glycLDL was prepared by incubating native low-density lipoprotein (nLDL) with 80 mmol/l glucose in sterile phosphate buffer containing 2 mmol/l EDTA, 1,000 UI/100 ml aprotinin, 100 μ M deferoxamine, and 0.009% gentamicin for 10 days at 37°C (21). We have previously shown that the extent of glycosylation achieved by this method is typically 10% to 12%, as measured immunoenzymatically (17). The nLDL was similarly incubated for 10 days at 37°C with sterile buffer but without glucose, which resulted in glycosylation of 1% to 1.5% (17). The nLDL and glycLDL (300 μ g/ml) were then each incubated for 12 h at 37°C with 1 μ M copper sulfate (17,23). The malondialdehyde concentration was measured by the thiobarbituric acid-reactive substances (TBARS) assay (17,23). Relative agarose mobility and trinitrobenzenesulfonic acid (TNBSA) reactivity served as markers of oxidatively modified proteins (17,23). Oxidized forms of LDL were then incubated for 24 h with endothelial cells at 37°C, under 95% air and 5% CO₂ (see subsequent text).

To further investigate the dose-dependent contribution of glycooxidative end products, we also prepared two oxidation-matched forms of lipoproteins (second form of oxLDL and glyc-oxLDL) containing similar amounts of TBARS and TNBSA reactivity by interrupting the incubation of glycLDL with the oxidant at an earlier time point (~10 to 12 h) (Table 1).

Cell culture. Human coronary endothelial cells were cultured using standard procedures, as described (6,8). The incubation medium (delipidated Dulbecco's Medium Essential Medium) was supplemented with 10 ng/ml human epidermal growth factor, penicillin/streptomycin, amphotericin B, and glutamine (6,8).

NOSIII bioactivity measurements. The effect of different forms of lipoproteins on NOSIII metabolism of ³H-arginine to ³H-citrulline was determined using standard

Table 1. Effects of Glycosylation and Oxidation on Low-Density Lipoprotein

	MDA (nmol/mg protein)	REM (cm)	TNBS (% Decrease)*
nLDL	2.0 \pm 0.6 (1.3–2.8)	1.2 \pm 0.2 (0.9–1.5)	—
glycLDL	4.0 \pm 1.2† (2.6–5.3)	1.3 \pm 0.2 (1.0–1.6)	—
oxLDL	28.9 \pm 4.2‡ (23.9–33.5)	2.3 \pm 0.3‡ (1.9–2.7)	29.6 \pm 5.6 (23.8–36.1)
glyc-oxLDL	40.4 \pm 4.1§¶ (35.1–45.3)	3.1 \pm 0.4§¶ (2.6–3.6)	42.6 \pm 4.8¶ (37.5–48.2)
oxLDL (second form)	25.4 \pm 3.3 (21.5–29.2)	—	27.8 \pm 3.8 (23.6–32.5)
glyc-oxLDL (second form)	24.8 \pm 3.9 (20.5–29.4)	—	25.9 \pm 4.1 (21.5–31.9)

*The decrease in TNBS reactivity in 18 h of oxLDL and glyc-oxLDL versus respective control agents in the absence of oxidant (mean \pm SD of 8 different experiments). †p < 0.05 vs. nLDL. ‡p < 0.001 vs. nLDL. §p < 0.001 vs. glycLDL. ¶p < 0.05 vs. oxLDL. ||p < 0.001 vs. respective nonoxidized control agents. The second forms of oxidized lipoproteins were prepared after exposure of lipoproteins to oxidants for ~10 to 12 h. Data are presented as the mean value \pm SD (intervals) of four different experiments: nLDL = native LDL; glycLDL = glycosylated LDL; oxLDL = oxidized LDL; glyc-oxLDL = glycosylated and oxidized LDL.

MDA = malondialdehyde; REM = relative electrophoresis mobility on agarose gel (cm from baseline); TNBS = trinitrobenzenesulfonic acid.

techniques, as described (25). Cell lysates (150 to 250 μ g protein) were incubated with the reduced form of nicotinamide adenine dinucleotide phosphate (reduced nicotinamide adenine dinucleotide phosphate; 2 mM), CaCl_2 (230 μ M), tetrahydrobiopterin (3 μ M), and ^3H -arginine (0.2 μ Ci, 10 μ M) for 20 min at 37°C. The assay volume was kept constant at 100 μ l. To determine whether glyc-oxLDL altered NOS activity, the assay was repeated with EDTA (1.7 mM) replacing calcium in the assay.

Western blot analysis. Whole-cell extracts were prepared by a modification of the standard procedure of Western blot analysis (6,8,26). Western blot analysis was performed with antibodies (1:500) against endothelial NOSIII (N-20, Santa Cruz, San Diego, California), with an epitope corresponding to an amino acid sequence mapped at the aminoterminal of NOSIII of human origin, without cross reactivity to NOSI or NOSII. The protein content of the extract was normalized with a polyclonal antibody against gamma-tubulin protein (Sigma-Aldrich, Milan, Italy). Semiquantitative densitometry of blots was performed using a Scan LKB (Pharmacia-Sweden, Bromma, Sweden) (6,8,17).

Northern blot analysis. Total ribonucleic acid (RNA) was isolated using spin columns (RNeasy, Qiagen Inc., Valencia, California). Agarose/formaldehyde gels were loaded with 10 μ g RNA per lane, and RNA was resolved by electrophoresis. The RNA was transferred to a nylon membrane (Hybond-N, Amersham, Piscataway, New Jersey) and cross-linked by ultraviolet radiation. The membrane was hybridized with radiolabeled alpha- ^{32}P deoxycytidine triphosphate (random prime kit, Boehringer, Munich, Germany), with the NOSIII fragment spanning between exon 3 and 4 (from nucleotides 1982 to 2703 of the human complementary deoxyribonucleic acid [cDNA] sequence) (27), and glyceraldehyde-3-phosphate dehydrogenase (Gene Bank, National Library of Medicine/NIH) cDNA in a hybridization solution (0.25 M Na_2PO_4 , pH 7.2, 1 mM EDTA, 20% sodium dodecyl sulfate). Hybridization was performed overnight at 65°C using 2×10^6 cpm/ml for each probe. To determine the effect of glyc-oxLDL on NOSIII messenger ribonucleic acid (mRNA) half-life, actinomycin D was used as an inhibitor of mRNA synthesis (28).

In vitro elongation of nascent RNA (run-on assay). Nuclei from 10^8 endothelial cells were prepared and in vitro transcribed with (^{32}P) uridine 5'-triphosphate, as described (29). The cDNA for NOSIII and beta-tubulin were used as probes. pBluScript I (Stratagene, Cedar Creek, Texas) plasmid deoxyribonucleic acid was used as a control agent.

Electrophoretic mobility shift assay (EMSA). A double-stranded probe (–1393 to –1374) containing the putative sterol-responsive element (SRE) present in the NOSIII promoter (28) was used as a probe in EMSA analysis. Nuclear extracts (10 μ g) were used for the shift assay, as described (7).

Determination of cyclic guanosine monophosphate (cGMP). Endothelial cells (10^6 cells) were incubated with 0.5 mmol/l isobutyl-1-methylxanthine at the time of glyc-

oxLDL addition and incubated with the various forms of LDL for 24 h. During the last 3 min, bradykinin (Sigma) at a final concentration of 3 nM was added, followed by extraction with trichloroacetic acid solution (30). The content of cGMP was measured using a specific immunoreactivity kit (Amersham), according to the manufacturer's recommendations.

Statistical analysis. Data are expressed as the mean value \pm SEM or, when specified, as the mean value \pm SD. Differences among the groups were tested by one-way analysis of variance, followed by the Bonferroni-corrected *t* test (two-hypothesis test), with significance accepted at $p < 0.05$.

RESULTS

Effects of glycosylation and oxidation on LDL. Table 1 shows the degree of lipoprotein oxidation with or without concurrent glycosylation. As previously demonstrated (17), oxidative modification of lipoproteins was enhanced by glycosylation: malondialdehyde and REM were increased and TNBSA decreased more in glyc-oxLDL than in oxLDL or glycLDL alone. In parallel experiments, two forms of oxidized lipoproteins (oxLDL and glyc-oxLDL) containing similar amounts of oxidation compounds were compared (Table 1); the second forms of oxLDL and glycLDL were similar in their malondialdehyde and TNBSA reactivity.

cGMP activity. As shown in Figure 1, we detected a marked dose-dependent reduction of cGMP levels in both rest and bradykinin-stimulated cells after exposure to glyc-oxLDL for 24 h. Under basal conditions (Fig. 1, upper panel), we observed reductions of 25% and 40% in cGMP levels at glyc-oxLDL doses of 30 μ g/ml ($p = \text{NS}$) and 100 μ g/ml, respectively ($p < 0.005$ vs. untreated cells, nLDL, glycLDL, and oxLDL), and a reduction of 67% at dose of 300 μ g/ml ($p < 0.01$ vs. all other forms). The nLDL and glycLDL did not show any effects on basal cGMP levels, whereas both forms of oxLDL and glyc-oxLDL (at doses of 100 μ g/ml) induced a reduction of 30% in cGMP ($p < 0.01$ vs. untreated cells, nLDL, and glycLDL). In bradykinin-stimulated cells (Fig. 1, lower panel), all forms of LDL blunted cGMP levels. The glyc-oxLDL reduced cGMP activity by 42% and 64% at doses of 30 and 100 μ g/ml, respectively ($p < 0.01$ vs. untreated, nLDL, glycLDL, and oxLDL), and by 82% at a dose of 300 μ g/ml ($p < 0.01$ vs. 30 and 100 μ g/ml of glyc-oxLDL). Hence, at equivalent doses of 100 μ g/ml, both forms of glyc-oxLDL induced a more pronounced attenuation of cGMP accumulation, as compared with either glycLDL or oxLDL.

NOSIII activity. The effects of glyc-oxLDL on NOSIII activity were further explored using the ^3H -citrulline assay. To maximize the detectability of its effects, high-dose glyc-oxLDL (300 μ g/ml) was used. However, even this dose of glyc-oxLDL did not significantly modify the ability of NOSIII protein to metabolize L-arginine to L-citrulline, as compared with 100 μ g/ml of nLDL and oxLDL ($56.5 \pm$

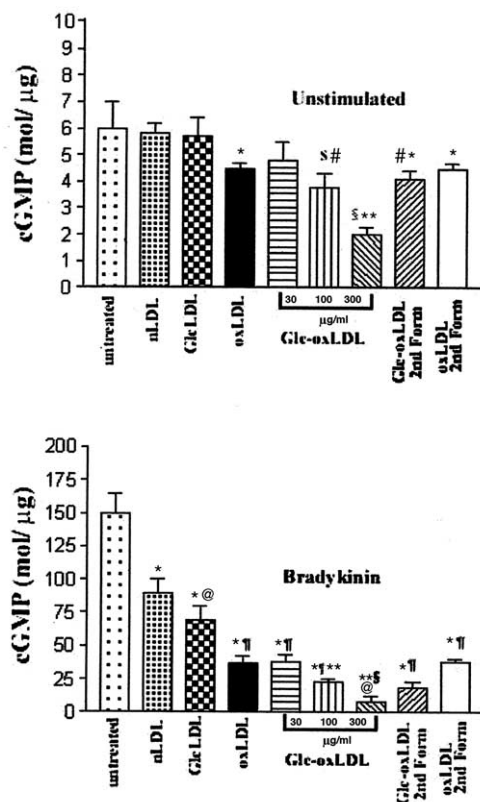


Figure 1. (Upper) Cyclic guanosine monophosphate (cGMP) levels in human endothelial cells after 24-h exposure to: native low-density lipoprotein (nLDL) (100 μ g/ml), glycosylated low-density lipoprotein (glcLDL) (100 μ g/ml), oxidized low-density lipoprotein (oxLDL) (100 μ g/ml), glc-oxLDL (30 μ g/ml), glc-oxLDL (100 μ g/ml), glc-oxLDL (300 μ g/ml), glc-oxLDL (second form) (100 μ g/ml), and oxLDL (second form) (100 μ g/ml). The 100- and 300- μ g/ml doses of glc-oxLDL reduced significantly cGMP activity. * $p < 0.05$ vs. untreated cells; $\#p < 0.01$ vs. untreated cells, nLDL and glcLDL; $\#p < 0.05$ vs. oxLDL; $\$p < 0.001$ vs. glc-oxLDL at 30 and 100 μ g/ml, second form of glc-oxLDL and second form of oxLDL; $\$p < 0.0001$ vs. untreated cells, nLDL and glcLDL. **(Lower)** The cGMP levels in cells stimulated with bradykinin and exposed for 24 h to the same lipoproteins as in the upper panel. * $p < 0.001$ vs. untreated cells; $@p < 0.01$ vs. nLDL; $†p < 0.001$ vs. nLDL and glcLDL; $**p < 0.001$ vs. oxLDL and glc-oxLDL at 30 μ g/ml and second form of oxLDL; $\$p < 0.0001$ vs. untreated cells, glcLDL, oxLDL, glc-oxLDL at 30 μ g/ml.

11.2 pmol citrulline [$p = 0.098$ vs. nLDL], 64.6 ± 9.4 mg protein, and 58.7 ± 8.9 min). In the presence of EDTA, NOSIII activity was almost undetectable.

Western blot analysis performed on whole-protein extracts showed that the amount of NOSIII protein significantly decreased after exposure of cells to glcLDL and glyc-oxLDL (Fig. 2). Although both forms of glcLDL and glyc-oxLDL decreased NOSIII protein compared with nLDL, at similar concentrations, glyc-oxLDL decreased it even further. Interestingly, when oxLDL and glyc-oxLDL were matched for oxidative modifications, downregulation of NOSIII protein was induced only by glyc-oxLDL, indicating that this was likely related to glycosylation and not merely due to different levels of oxidative compounds (Fig. 2). These phenomena were consistent with the downregulation of NOSIII mRNA expression observed using

Northern blot analysis (Fig. 3). Densitometric analysis of four pooled experiments showed a significant decrease in mRNA for NOSIII induced by oxLDL, glcLDL, and glyc-oxLDL. Longer exposure periods (48 to 96 h) did not result in a further decrease (data not shown).

NOSIII mRNA stability. We investigated the effects of glyc-oxLDL on the half-life of NOSIII mRNA. Cells were cultured for 24 h in the presence or absence of 300 μ g/ml glyc-oxLDL, and actinomycin D was then added to the cells to further inhibit transcription. At different time points after the addition of actinomycin D, total RNA was isolated and examined by Northern blot analysis. The decay of NOSIII mRNA from untreated cells and cells incubated with glyc-oxLDL for 24 h was similar (Fig. 4), indicating that even at a relatively high dose, glyc-oxLDL did not influence the mRNA half-life. Indeed, glyceraldehyde-3-phosphate dehydrogenase mRNA levels did not change throughout the experiments (Fig. 4). Similarly, all remaining forms of lipoproteins did not affect the mRNA half-life until a dose of 1 mg/ml was used (data not shown).

Transcription rate of the NOSIII gene. To ascertain whether glyc-oxLDL attenuated nascent NOSIII mRNA formation, a nuclear run-on experiment was performed. As shown in Figure 5, glyc-oxLDL dose-dependently decreased the rate of NOSIII gene transcription directly in the nucleus. Densitometric analysis revealed that glyc-oxLDL and its second form (at dose of 100 μ g/ml) induced a nonsignificant reduction of $\sim 5\%$ in NOSIII transcript and a significant reduction of $\sim 25\%$ at dose of 300 μ g/ml ($p < 0.05$ vs. untreated cells, nLDL, glcLDL, and both forms of oxLDL), although neither glcLDL nor the two forms of oxLDL significantly affected the nascent NOSIII gene transcript (Fig. 5).

SRE. To further explore whether glyc-oxLDL interfered with the regulatory elements present in the NOSIII promoter, SRE was analyzed. The EMSA detects specific DNA-protein complexes between nuclear extracts from cells and the probe containing the SRE sequence. Cells were incubated with nLDL, glcLDL, oxLDL, and glyc-oxLDL at different concentrations, as indicated in Figure 6. The glyc-oxLDL, but neither nLDL nor glcLDL nor oxLDL, induced a significant reduction of the retarded band.

DISCUSSION

This study demonstrates, for the first time, that concurrent glycosylation and oxidation of LDL induced and amplified potent downregulation of NOSIII in human coronary endothelial cells. We have found that exposure of cells to glyc-oxLDL for 24 h resulted in a decrease in both NOSIII mRNA and protein.

The oxLDL reduced NOSIII expression, decreasing its protein and mRNA levels in endothelial cells from human saphenous veins (29). Several stimuli, such as tumor necrosis factor- α , lipopolysaccharide (30–32), and albumin-derived AGEs (28) also downregulate NOSIII (protein

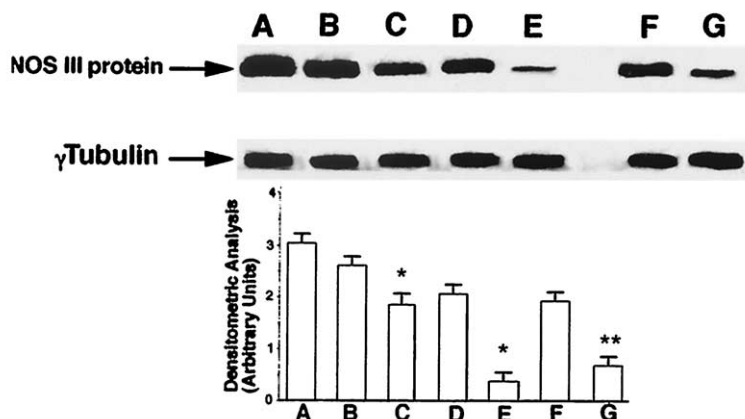


Figure 2. (Top) Western blot of endothelial nitric oxide synthase III (NOSIII) in cells exposed to the following conditions: **A** = untreated cells; **B** = native low-density lipoprotein; **C** = glycosylated low-density lipoprotein (glycLDL); **D** = oxidized low-density lipoprotein (oxLDL); **E** = glyc-oxLDL; **F** = oxLDL (second form); and **G** = glyc-oxLDL (second form). All concentrations are 100 μ g/ml. (Bottom) Densitometric analysis of endothelial NOSIII levels (mean \pm SD) representative of six separate experiments. Endothelial NOSIII was significantly decreased in **C** (* p < 0.01 vs. **B**) and **E** (# p < 0.0001 vs. **B** and **C**). Similarly, **G** was significantly decreased compared with **F** (** p < 0.0004 vs. **F**).

and mRNA) in bovine aortic endothelial cells. The AGEps downregulate NOSIII without interfering with its transcriptional rate (28), and the major mechanism involved seems to be the enhanced degradation rate of NOSIII mRNA, thereby reducing its half-life (31). Destabilization of NOSIII mRNA by AGEp-bovine serum albumin could result from a protein-mRNA interaction, which would allow the degradation elements in the 3' untranslated region to become active (33).

In contrast, the effects of glyc-oxLDL on decreasing NOSIII mRNA do not seem to be post-transcriptional, because experiments with actinomycin D did not show any effect on NOSIII mRNA stability, as demonstrated previously for nLDL (33,34). However, the present study demonstrates that glyc-oxLDL does interfere, at least in part, with the rate of NOSIII gene transcription. Run-on experiments (detecting the amount of nascent mRNA synthesis) showed that high-dose glyc-oxLDL reduces the formation of nascent RNA. We have also shown that SRE, which is present in the 5'-flanking region of human NOSIII (35), could be involved in the regulation of NOSIII transcription. Indeed, the EMSA experiments (detecting specific DNA-protein complexes between cell nuclear extracts and the probe containing the SRE) revealed a clear dose-dependent decrease in the retarded band after 24-h exposure to glyc-oxLDL. This decrease was obvious with 100 μ g/ml of

glyc-oxLDL and achieved a maximal 25% reduction at a dose of 300 μ g/ml; nLDL and glycLDL at doses of 100 μ g/ml did not exert any effect. No significant decrease in the retarded band was observed after exposure to either form of oxLDL, suggesting that the SRE-protein complexes are specifically reduced by glyc-oxLDL.

The reduction of NOSIII mRNA by glyc-oxLDL required a 24-h incubation period, which is a long lag time for simple downregulation at the transcriptional level, due to a reduction of the NOSIII promoter at the binding site. For this reason, we cannot exclude the possibility that a more complex mechanism might be involved in transcriptional regulation of NOSIII by glyc-oxLDL in vivo. Moreover, it is highly likely that another mechanism, such as an increase of mRNA degradation, may play a role. Results obtained with oxidation-matched forms of lipoproteins (oxLDL and glyc-oxLDL, second forms) indicated that downregulation of NOSIII protein and mRNA by glycoxidation was not merely due to an increase in oxidative compounds.

Furthermore, we have shown that these effects of glyc-oxLDL on NOSIII were functionally significant, because exposure of cells to glyc-oxLDL for 24 h resulted in a dose-dependent decrease in cGMP levels. We have also observed that in the absence of LDL, bradykinin stimulated cells and enhanced cGMP activity, in agreement with a previous study (36). Both glyc-oxLDL and oxLDL reduced the absolute levels of cGMP, but at comparable concentrations, the effects of glyc-oxLDL were appreciably greater. In addition, the conversion of 3 H-arginine into 3 H-citrulline, under apparent V_{max} conditions (25,37), is one of the measures of NOSIII bioactivity. Under our experimental conditions, glyc-oxLDL did not significantly modify the inherent capability of NOSIII to metabolize L-arginine to L-citrulline any more than nLDL or oxLDL. Our results are consistent with those obtained with oxLDL in human endothelial cells (38), but not with those obtained at high

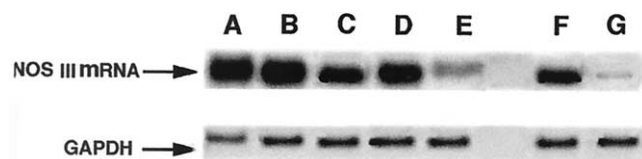


Figure 3. Northern blot of endothelial nitric oxide synthase III (NOSIII) in human endothelial cells. **A** = untreated cells; **B** = nLDL; **C** = glycLDL; **D** = oxLDL; **E** = glyc-oxLDL; **F** = oxLDL (second form); and **G** = glyc-oxLDL (second form). All concentrations are 100 μ g/ml. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; mRNA = messenger ribonucleic acid. Other abbreviations as in Figure 2.

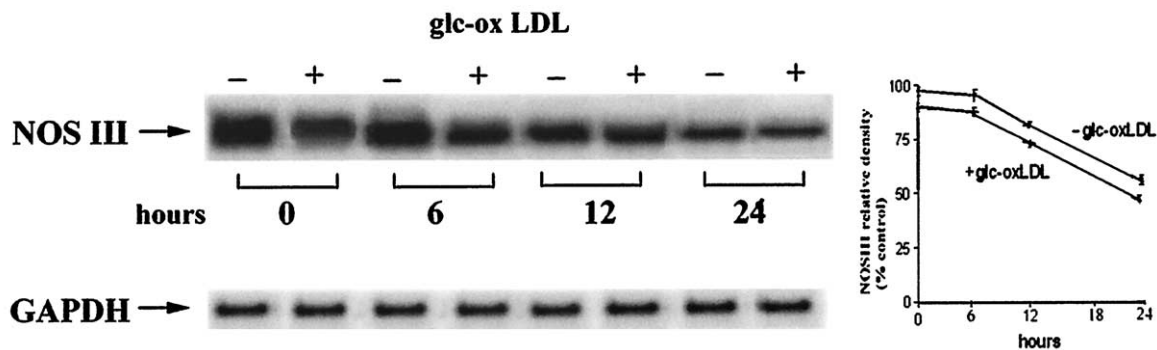


Figure 4. Cells treated with 3 nM of actinomycin D (to inhibit further transcription) in the absence (–) or presence (+) of glyc-oxLDL (300 µg/ml) for 24 h before harvesting ribonucleic acid. A representative Northern blot hybridized with a specific probe for NOSIII and GAPDH is shown. The relative percentage of NOSIII signal is plotted against time. The results did not show any difference between the treatments. Each point represents the mean value \pm SEM for three experiments. Abbreviations as in Figures 2 and 3.

doses of hypochlorite-modified LDL in bovine aortic endothelial cells (39). Taken together, the present study indicates that at least one of the mechanisms involved in downregulation of NOSIII by glyc-oxLDL may be a decrease in the transcription rate due to reduced binding of transcription factors to SRE—effects that subsequently impact intracellular cGMP levels.

Glycation, per se, has been shown to enhance LDL oxidation and contribute to the acceleration of atherosclerosis (17,18,40–42). Similarly, the presence of high levels of glycoxidation and AGEs in serum and tissues, in association with atherosclerosis, diabetes, renal failure, and aging, has been well documented (14–16,19–22,43–46). Although the concentrations of glyc-oxLDL used in the present study are higher than those usually encountered in

the plasma of diabetic patients (1,14–16,21), much higher concentrations of glyc-oxLDL can be found in poorly controlled diabetic patients (47) and elderly diabetic patients (47). Thus, we suggest that long-term exposure to relative high-dose glyc-oxLDL (and AGEs) can conceivably induce significant effects on NOSIII activity in such patients. Moreover, the major chemical glycoxidation products of AGEs (e.g., *N*-carboxymethyl lysine and pentosidine) were found in human atherosclerotic lesions (19,40–42,48). Endothelial dysfunction is an early event during atherogenesis (12,13), and the effects on NOSIII seen in the present study suggest that endothelial dysfunction during diabetes mellitus may be related to glyc-oxLDL-mediated signaling alterations. Furthermore, vascular smooth muscle cell proliferation is clearly associated with the accelerated diabetic

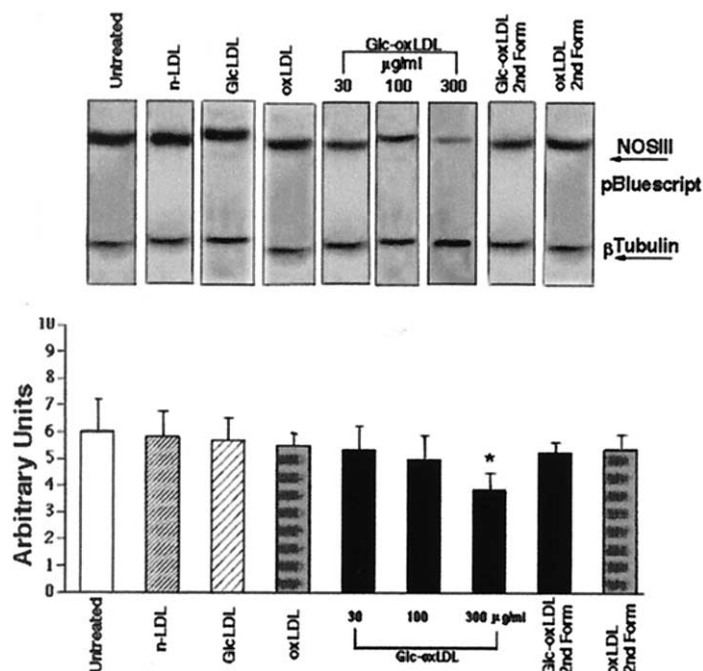


Figure 5. In vitro elongation of nascent ribonucleic acid (run-on assay) performed in human endothelial cells exposed to following conditions: 100 µg/ml of nLDL, glycLDL, or oxLDL; glyc-oxLDL at 30, 100, and 300 µg/ml; and 100 µg/ml of the second forms of glyc-oxLDL and oxLDL. Densitometric analysis revealed a 25% reduction in NOSIII transcript after treatment with 300 µg/ml of glyc-oxLDL (**p* < 0.05 vs. all other forms). Abbreviations as in Figures 2 and 3.

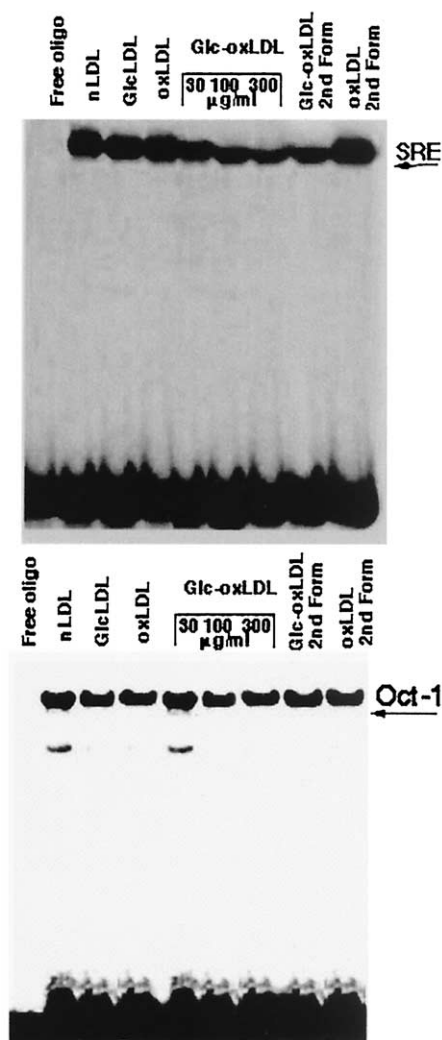


Figure 6. Analysis of putative sterol-responsive element (SRE) present in the NOSIII promoter by electrophoretic mobility shift assay (EMSA). **(Upper)** Representative EMSA was performed with a ^{32}P end-labeled probe containing the SRE element and nuclear extracts from cells treated for 24 h with nLDL (100 $\mu\text{g}/\text{ml}$), glycLDL (100 $\mu\text{g}/\text{ml}$), oxLDL (100 $\mu\text{g}/\text{ml}$), glyc-oxLDL (30 $\mu\text{g}/\text{ml}$), glyc-oxLDL (100 $\mu\text{g}/\text{ml}$), glyc-oxLDL (300 $\mu\text{g}/\text{ml}$), glyc-oxLDL (100 $\mu\text{g}/\text{ml}$, second form), and glyc-oxLDL (100 $\mu\text{g}/\text{ml}$, second form). **(Lower)** The EMSA was performed with the Oct-1 element used as a control agent to normalize the results (as described in [55]) and with a nuclear extract from cells treated for 24 h with the same lipoproteins. Abbreviations as in Figure 2.

vasculopathy. Nitric oxide mediates cytothasis of subendothelial smooth muscle cells (48,49), and AGEs may favor cell proliferation by quenching endothelium-derived NO in proliferative diabetic vascular lesions (39,50), playing a significant role in their onset. Thus, glyc-oxLDL may contribute to proliferative vasculopathy through interference with the NO-signaling pathway. Because NOSIII is localized in regions of the membrane identified as caveolae (51,52), further studies are needed to address the possible effects of glyc-oxLDL on caveolar rearrangements. Moreover, newly developed NO donors should be investigated in advancing vascular diseases associated with diabetes (53).

Thus, concurrent glycosylation and oxidation of LDL

have a profound impact on downregulation of NOSIII activity in human endothelial cells. Notably, gene transfer of NOSIII improves relaxation of carotid arteries from diabetic rabbits (54), supporting a physiologically significant role for this pathway in the vascular regulation of diabetes. Although cell-culture studies may indeed bear only partial relevance to pathophysiologic mechanisms activated *in vivo*, glycoxidation and NO-related cascade of events may, in turn, influence the evolution of atherogenesis, coronary heart disease, and other vascular complications in diabetic patients, especially in poorly controlled and/or elderly patients.

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